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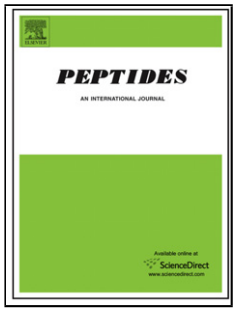

Title: Nesfatin-1 modulates murine gastric vagal afferent mechanosensitivity in a nutritional state dependent manner

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Title: Nesfatin-1 modulates murine gastric vagal afferent mechanosensitivity in a nutritional state dependent manner.

Short title: Nesfatin-1 and gastric vagal afferents

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Highlights

- Nesfatin-1 potentiated mucosal gastric vagal afferent responses to mucosal stroking in lean mice fed ad libitum or fasted overnight, an effect not observed in high fat diet mice.
- Gastric vagal tension receptor mechanosensitivity was unaffected by nesfatin-1 in lean mice fed ad libitum or fasted overnight, but was inhibited in high fat diet mice.
- Plasma concentrations of nesfatin-1were unchanged between standard laboratory diet mice fed ad libitum or fasted overnight and high fat diet fed mice.
- The precursor for nesfatin-1 (NUCB2 mRNA) was detected in the gastric mucosa and gonadal fat with no difference in expression between fed ad libitum, fasted and high fat diet fed groups.
- Nesfatin-1 modulated gastric vagal afferents in a nutritional state dependent manner.

Abstract

Food intake is regulated by vagal afferent signals from the stomach. Nesfatin-1 is an anorexigenic peptide produced within the gastrointestinal tract and has well defined central effects. We aimed to determine if nesfatin-1 can modulate gastric vagal afferent signals in the periphery and further whether this is altered in different nutritional states. Female C57BL/6J mice were fed either a standard laboratory diet (SLD) or a high fat diet (HFD) for 12 weeks or fasted overnight. Plasma nucleobindin-2 (NUCB2; nesfatin-1 precursor)/nesfatin-1 levels were assayed, the expression of NUCB2 in the gastric mucosa and adipose tissue was assessed using real-time quantitative reverse-transcription polymerase chain reaction. An *in vitro* preparation was used to determine the effect of nesfatin-1 on gastric vagal afferent mechanosensitivity. HFD mice exhibited an increased body weight and adiposity. Plasma NUCB2/nesfatin-1 levels were unchanged between any of the groups of mice. NUCB2 mRNA

was detected in the gastric mucosa and gonadal fat of SLD, HFD and fasted mice with no difference in mRNA abundance between groups in either tissue. In SLD and fasted mice nesfatin-1 potentiated mucosal receptor mechanosensitivity, an effect not observed in HFD mice. Tension receptor mechanosensitivity was unaffected by nesfatin-1 in SLD and fasted mice, but was inhibited in HFD mice. In conclusion, Nesfatin-1 modulates gastric vagal afferent mechanosensitivity in a nutritional state dependent manner.

Abbreviations: CCK, cholecystokinin; HFD, high fat diet; 5-HT, 5-hydroxytryptamine; NUCB2, nucleobindin-2; QRT-PCR, real-time quantitative reverse-transcription polymerase chain reaction; SLD, standard laboratory diet;

Keywords

Nesfatin-1, obesity, vagal afferents

1. Introduction

Gastric vagal afferents are a vital peripheral mechanism by which the gastrointestinal tract can communicate with the CNS to modulate food intake as well as gastrointestinal function. Within the stomach of mice there are two populations of mechanosensitive vagal afferent [33]. These can be discriminated based on their responses to mechanical stimuli [33]. The first are tension sensitive afferents which have a well characterized role, detecting distension and contraction of the stomach wall, and can activate satiety circuitry in the brain [52]. The second class are mucosal afferents and whilst they are well defined morphologically and electrophysiologically, their role in modulating physiological function is less clear [2-4]. Limited evidence suggest a

role for these afferents in detecting food particle size within the lumen to provide negative feedback on the control of gastric emptying [1].

The gastrointestinal tract is littered with discrete cell populations which contain peptides and other chemicals which have the ability to modulate vagal afferent firing and also regulate food intake [10]. These peptides include classic mediators of food intake such as leptin [23], ghrelin [21], 5-hydroxytryptamine (5-HT), cholecystokinin (CCK) [11, 42] and adiponectin [25]. In addition, there are substantial amounts of nesfatin-1 located within discrete vesicles in X/A-like cells within the stomach [48]. These specialised X/A-like endocrine cells also produce the orexigenic peptide ghrelin [48].

The nesfatin-1 peptide is a relatively recent discovery. It was originally identified as a hypothalamic satiety factor being present in the paraventricular nucleus where it has been shown to reduce food intake in a melanocortin 3/4 receptor dependent and leptin independent manner [32]. Nesfatin-1 has also been shown to potentiate the excitatory and inhibitory effect of glucose on sub-populations of glucose sensing neurons in the dorsal vagal complex [13]. In addition, it is present in neurons of the nucleus tractus solitaries activated by gastric distension [6]. Nesfatin-1 has also been shown to activate isolated CCK and capsaicin sensitive vagal afferent neurons by increasing Ca^{2+} influx [18]. Additionally, peripheral administration of the active fragment of nesfatin-1 fails to reduce food intake in mice that have been treated with capsaicin [46]. Together this evidence suggests a possible vagal route of action for nesfatin-1 to regulate food intake. However, to date, there have been no studies to establish the effect of nesfatin-1 on gastric vagal afferent endings and whether this may represent a peripheral mechanism by which nesfatin-1 could modulate food intake.

Peptidergic signalling is known to be sensitive to nutritional status and nesfatin-1 is no exception. Gastric nucleobindin 2 (NUCB2, precursor for nesfatin-1) content has been shown to be decreased in fasting [48]. Diet induced obesity is also associated with altered levels of appetite regulating peptides including leptin, ghrelin, peptide YY [49] and NUCB2/nesfatin-1 [31]. Furthermore, expression of receptors for gut peptides is altered in the cell bodies of vagal afferents within the nodose ganglia in fasted and diet induced obese rats and mice [34]. Gastric vagal afferent mechanosensitivity responses to peptides are also altered in obesity. For example, the effect of leptin on gastric vagal afferents switches from potentiation of mucosal receptors to an inhibition of tension receptors in obesity [24]. Thus, it needs to be determined whether the effects of nesfatin-1 on vagal afferent function depend on nutritional status.

Through the use of *in vitro* electrophysiology we sought to identify the effect of nesfatin-1 on gastric vagal afferent mechanosensitivity in lean, obese and fasted mice to determine a possible role in the modulation of gastric vagal afferent mediated physiological functions under different states of nutrition.

2. Materials and Methods

2.1 Ethical approval

All experimental protocols were approved by the animal ethics committees of the Institute of Medical and Veterinary Science, South Australian Health and Medical Research Institute (SAHMRI) and the University of Adelaide and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Mouse study groups

Thirty 7 week old female C57BL/6J mice (Animal Resource Centre, Perth, Australia) were acclimatized for 1 week before being randomly divided into two groups and fed either a high fat diet (HFD, N=18) comprising 60%, 20%, 20% of energy from fat, protein, and carbohydrate (Adapted from Research Diets Inc., New Brunswick, USA) or a standard laboratory diet (SLD, N=12) comprising 12%, 23%, 65% of energy from fat, protein and carbohydrate (Specialty Feeds, Glen Forest, Western Australia) respectively for 12 weeks. A maximum of four mice were housed per cage and were kept under 12 hour light: dark cycles with *ad libitum* access to food and water. A separate group of age matched female mice (N=6), fed a SLD for 12 weeks at the same time and under identical conditions to the other two groups, were fasted overnight prior to experimentation.

2.3 In vitro mouse gastric vagal afferent preparation

After the completion of the 12 week diet period mice fed *ad libitum* or fasted overnight were euthanized between 0600 and 0900hr, via 4% isoflurane anaesthetic and exsanguination through the abdominal aorta followed by decapitation. The stomach and oesophagus including both vagal nerves were dissected as previously described [21]. In short, the stomach and oesophagus, with intact vagal nerves, were removed. The stomach and oesophagus were opened out longitudinally mid-way between the two main vagal branches, either side of the oesophagus, and along the greater curvature of the stomach. The ventral half of the stomach was removed and kept for the real-time quantitative reverse-transcription polymerase chain reaction (QRT-PCR) experiments described below. The dissection process was carried out at 4°C in modified Krebs solution composing of (in mM): 118.1 NaCl, 4.7 KCl, 25.1 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4.7H_2O$, 1.5 $CaCl_2$, 1.0 citric acid, 11.1 glucose and 0.001 nifidipine, bubbled with 95% O_2- 5% CO_2. The tissue was then pinned down mucosa side up in a purpose built organ bath and superfused with the oxygenated modified Krebs solution. The vagal

afferent nerves were passed through into an adjacent paraffin oil filled chamber for electrophysiology recording.

2.3.1 Characterization of gastric vagal afferent properties

The location of individual receptive fields corresponding to afferent fibres was identified by mechanical stimulation of the mucosa with a soft brush. The sub-type of each afferent was then further determined by responses specific to each sub-type of gastric vagal afferent mechanoreceptor, as previously described [21]. Since the mucosal gastric vagal afferent receptive field is generally very small (≈1mm^2), the response of the middle 8 of 10 stimulations was averaged to minimize error. For tension receptors, circular tension was applied by a hook, placed adjacent to the receptive field, attached to a cantilever via suture silk. Weights were applied in a step-wise manner from 1-5g for 1min per weight and the response measured as mean discharge frequency over the 1min period. A recovery period of 1min was allowed between the applications of each tension stimuli.

2.3.2 Effect of nesfatin-1 on the mechanosensitivity of gastric vagal afferents

Following the establishment of gastric vagal afferent baseline mechanosensitivity, the effect of nesfatin-1 on the mechanosensitivity was determined. Nesfatin-1 (30pM), at a concentration previously reported in the literature [9], was added to the modified Krebs, superfused into the organ bath and allowed to equilibrate for 20min to ensure penetration of all layers of the gastric tissue before vagal afferent mechanosensitivity was re-determined. This procedure was repeated for higher doses of nesfatin-1 (100 and 300pM). Time control experiments were utilized to ensure that no significant change in mechanosensitivity occurred over a comparable duration independent of addition of nesfatin-1.

2.4 Quantification of plasma NUCB2/nesfatin-1

Plasma samples extracted from the blood collected from the abdominal aorta during euthanasia, between 0600 and 0900hr, were analysed in duplicate for circulating NUCB2/nesfatin-1 levels using a Phoenix Peptides Nesfatin-1 (1-82) (rat) ELISA kit (EK-003-22)[31] as per the manufacturer's instructions (Phoenix Peptides, Burlingame, CA, USA) using the same assay kit. Quantification was measured at 450nm. The NUCB2/nesfatin-1 assay has a detection threshold of 1.26ng mL^{-1}, with a detectable range from 0.1-1000 ng mL^{-1}. The amount of immunoreactive material was determined using a non-linear regression curve-fit. This was used to quantify and compare the concentration of NUCB2/nesfatin-1 in the plasma samples. Plasma concentrations of NUCB2/nesfatin-1 were not obtained for all of the mice studied due to the fact that an insufficient quantity of plasma was collected from some mice.

2.5 Real-time quantitative reverse-transcription polymerase chain reaction (QRT-PCR)

During the dissection, for the *in vitro* gastric vagal afferent preparation, the gastric mucosa was removed from the unused ventral half of the stomach by gently scrapping the mucosa from the muscular layers using a scalpel blade. The gastric mucosa was snap frozen in liquid nitrogen and stored at -80°C until required. Total RNA was then extracted from the mucosa using a PureLink® RNA Mini Kit (12183018A, Ambion (Thermo Fisher, Scoresby, VIC, Australia). The gonadal fat pads were also collected during the dissection for the in vitro gastric vagal afferent preparation. They were weighed and snap frozen in liquid nitrogen prior to storage at -80°C. RNA was extracted from the fat using a TRIzol® Plus RNA Purification Kit (12183555, Ambion (Thermo Fisher)), in accordance with the manufacturer's instructions. The RNA yield and quality were quantified on a NanoDrop™ Lite spectrophotometer (Thermo Fisher) with an $OD_{260/280}$ in the range of 1.8 – 2.0 accepted as sufficiently pure for downstream applications.

For evaluation of NUCB2 expression, in stomach mucosa and white adipose tissue from SLD, HFD and fasted mice, QRT-PCR reactions were performed as previously described [28], using a 7500 Fast Real-time PCR System (Applied Biosystems® (Thermo Fisher)) and analysed with DataAssist™ Real-Time PCR Analysis Software (Applied Biosystems®). Samples were converted to cDNA using SuperScript® III First-Strand Synthesis SuperMix (18080400, Invitrogen (Thermo Fisher)). Quantification reactions were performed in triplicate according to the manufacturer's specifications with a TaqMan® Fast Advanced Master Mix (4444964, Applied Biosystems (Thermo Fisher)), incorporating specific pre-made TaqMan® gene expression assays (Applied Biosystems (Thermo Fisher)) for the detection of NUCB2 (Mm01137144_m1), β-actin (Actb; Mm00607939_s1), hypoxanthine guanine phosphoribosyl transferase (Hprt; Mm00446968_m1), β-2 microglobulin (B2m; Mm00437762_m1), 18S ribosomal (Rn18s; Mm03928990_g1) and peptidylprolyl isomerase A (Ppia; Mm02342429_g1) mRNA. Five housekeepers were tested for stability in stomach scrapings and fat from SLD, HFD and fasted mice. Normfinder stability values [35] indicate (Table 1) that Ppia, Hprt and B2m were appropriate stable housekeepers for stomach scraping samples, whereas, Ppia, actb and B2m were more appropriate for adipose tissue. Relative mRNA transcript expression was calculated as previously described [37] and normalised to the housekeepers Ppia, Hprt and B2m for stomach scrapings and Ppia, Actb and B2m for adipose tissue. No template controls for each gene expression assay were also performed.

2.6 Drugs

Nesfatin-1 (1μM; SRP4914, Sigma-Aldrich, Castle Hill, NSW, Australia) was kept frozen (-80°C) and used freshly diluted for each experiment.

2.7 Statistical analysis

All data in graphs are expressed as mean ± SEM with N= the number of individual animals used. Vagal afferent stimulus-response curves and weight change were analysed using two-way analysis of variance (two-way ANOVA) and Sidak post hoc tests. The effect of nutritional state on the modulatory action of nesfatin-1 on responses to mechanical stimulation was determined by assessing the response to either stroking (200mg von Frey hair, mucosal receptors) or tension (3g, tension receptors) at the different concentrations of nesfatin-1 (30-300pM). Significant difference in the effects of nesfatin-1 between the different fed states was assessed using two-way ANOVA with Sidak's post-hoc test. NUCB2 mRNA levels and plasma NUCB2/nesfatin-1 levels were analysed using a one-way ANOVA with Tukey's post-hoc test. Fat mass and final body weight was assessed using unpaired t-tests. Significance was defined at $P<0.05$.

3. Results

3.1 Effect of long-term alterations in diet on body composition

Mice on the HFD gained 54.94 ± 8.13% of their initial weight which was significantly more than the 25.88 ± 1.73% gained by the SLD mice (Fig. 1A, $P<0.001$ vs SLD, two-way ANOVA, Sidak post hoc test) causing them to be significantly heavier at the end of the 12 week diet period (SLD: 22.65±0.50g, HFD: 29.06±1.73g, Fig. 1B, $P<0.01$, unpaired t-test). The HFD mice also had increased gonadal adiposity of 1.29±0.24g compared to the SLD mice with 0.22±0.07g (Fig. 1C, $P<0.01$, unpaired t-test). The stomach content at the point of tissue collection was significantly greater in the SLD (0.2256±0.03g (n=12)) and HFD (0.1761±0.03g (n=18)) *ad libitum* fed mice compared to the mice fasted overnight (0.0356±0.01g (n=6); data not shown, $P<0.01$, one-way ANOVA, Tukey post-hoc test). There was no significant difference in stomach content between SLD and HFD *ad libitum* fed mice. Plasma NUCB2/nesfatin-1 was similar in SLD (6.11±0.37ng mL^{-1}), HFD (5.53±0.31ng mL^{-1}) and fasted (5.64±0.59ng mL^{-1}) mice (Fig. 1D, $P>0.05$, one-way ANOVA, Tukey post-hoc test).

3.2 NUCB2 expression in gastric mucosa and gonadal fat

QRT-PCR was used to determine the expression of NUCB2 in the gastric mucosa (Fig 2A) and gonadal white adipose tissue (Fig. 2B). There were no significant differences in the abundance of NUCB2 mRNA detected between any of the mouse groups ($P>0.05$, one-way ANOVA).

3.3 Effect of long-term alterations in diet on gastric vagal afferent response to nesfatin-1

3.3.1 Mucosal Receptors

In SLD mice nesfatin-1 dose-dependently potentiated the response of mucosal receptors to mucosal stroking with calibrated von Frey hairs (Fig 3A, 10-1000mg, $P<0.05$, two-way ANOVA, nesfatin-1 effect). In animals that had been fasted overnight nesfatin-1 still potentiated mucosal receptor mechanosensitivity (Fig 3B, 30pM: $P<0.05$, 100pM: $P<0.001$, 300pM: $P<0.01$, two-way ANOVA, nesfatin-1 effect). In mice that had been fed the HFD for 12 weeks (Fig. 3C) nesfatin-1 had no effect on mucosal receptor mechanosensitivity ($P>0.05$, two-way ANOVA, nesfatin-1 effect). When the percentage increase in the response to mucosal stroking with a 200mg von Frey hair was plotted against the concentration of nesfatin-1 for both SLD and fasted mice nesfatin-1 had a significant potentiating effect on mucosal receptor mechanosensitivity (Fig. 3D, both $P<0.01$ vs. HFD, two-way ANOVA, nutritional status effect) compared to the HFD mice where there was no effect of nesfatin-1. There was no significant difference between the nesfatin-1 induce potentiation observed in the SLD mice and the fasted mice ($P>0.05$, two-way ANOVA, nutritional state effect).

3.3.2 Tension Receptors

In the SLD (Fig. 4A) and fasted mice (Fig. 4B) nesfatin-1 had no effect on tension receptor mechanosensitivity (both $P>0.05$, two-way ANOVA, nesfatin-1 effect). However, nesfatin-1 significantly reduced the response of gastric tension receptors to mechanical stretch in mice fed the HFD for 12 weeks (Fig. 4C; $P<0.05$ vs. control, two-way ANOVA, nesfatin-1 effect). Analysis of the percentage inhibition of the response to 3g stretch caused by each concentration of nesfatin-1 in the three groups of mice revealed a significant gain in the inhibitory effect of nesfatin-1 on tension receptors in HFD fed mice (Fig 4D, $P<0.05$ vs SLD, two-way ANOVA, nutritional state effect), an effect absent in both the SLD and fasted mice.

4. Discussion

We demonstrate that nesfatin-1 has the ability to potentiate the mechanosensitivity of mucosal gastric vagal afferents in lean animals and that this ability is lost in HFD induced obesity and fasting. In addition, nesfatin-1 inhibited tension receptor mechanosensitivity in HFD mice.

In the current study we demonstrate that chronic HFD feeding for 12 weeks had no effect on gastric NUCB2 mRNA expression. In contrast, it has been demonstrated that feeding mice a HFD, identical to the diet used in the current study, for a longer period of time (17 weeks) caused a reduction in gastric NUCB2 mRNA levels [31]. However acute HFD feeding, *in vivo,* had no effect on gastric NUCB2 expression [31]. It is possible that the reduction in NUCB2 mRNA expression is only apparent after extensive exposure to a HFD, longer than the 12 weeks of the current study. The reduction in NUCB2 mRNA expression could be specific to the type of fatty acid that is consumed because it has previously been demonstrated that treatment with oleic acid, but not octanoic or linolenic acid, caused a reduction in NUCB2 mRNA expression in cell lines (MGN3-1 cells) [31]. The HFD used in both the current study and the study by Mohan *et al.* utilised lard as the fat source which contains a dominant amount

of oleic acid. Therefore the reduction in gastric NUCB2 expression observed after 17 weeks could be due to chronic exposure to oleic acid.

Adipose tissue nesfatin-1 protein levels have been shown to be increased in HFD fed mice and reduced in fasted mice [41]. Our results indicate a potential for increased NUCB2 mRNA in the gonadal adipose tissue which would be consistent with these results. However, there was considerable variation in NUCB2 between samples which prevented statistical significance from being reached. Plasma nesfatin-1 has previously been shown to be decreased in HFD mice [8]. We observed no change in plasma NUCB2/nesfatin-1 levels in mice fed a HFD or food restricted compared to mice fed a SLD. This discrepancy could be due to differences in diet duration (4 weeks in [8]) as well as potential sex differences in the regulation of nesfatin-1 levels which have previously been reported [16]. In addition, in the current study HFD mice were fed *ad libitum*, whereas, in the previous report [8], the decrease in plasma nesfatin concentration, in HFD mice, was observed in mice fasted for 6 hours [8]. Therefore the different feeding states could also explain the discrepancy. In humans there is similar controversy with some studies reporting a positive linear correlation [41, 50], others reporting no correlation [17, 30], and another study reporting a negative correlation [51] between BMI and plasma NUCB2/nesfatin-1.

Potentiation of mucosal receptor mechanosensitivity by nesfatin-1 may represent a peripheral mechanism by which nesfatin-1 can influence food intake. Whilst there is no direct evidence for gastric mucosal receptors in the control of food intake, their activation may lead to delayed gastric emptying, which can indirectly regulate food intake. Centrally administered nesfatin-1 has been shown to delay gastric emptying [47]. In addition, release of nesfatin-1 from adipocytes has been shown to be triggered by increasing glucose levels [15]. Increased glycaemia leads to delayed gastric emptying [44]. Thus, it is possible that after the ingestion

of a meal, increased circulating glucose may trigger the release of nesfatin-1 from adipocytes and perhaps gastric X/A cells, which could act locally to reduce gastric emptying, retaining food in the stomach longer. This hypothesis is highly speculative and requires further investigation.

A nesfatin-vagal pathway for the regulation of food intake is controversial. In rats, intracerebroventricular administration of nesfatin-1 (0.3-0.9nmol/rat) causes a reduction in food intake [39]. In contrast, peripheral administration of nesfatin-1 (8-73nmol/Kg) has been shown to have no effect on food intake despite being administered at doses 30 times higher than the intracerebroventricular administered nesfatin-1 [39]. In mice, there have been mixed reports for a peripheral effect of nesfatin-1 on food intake with one study showing no effect [14] and another showing a dose-dependent decrease in food intake [45] after intraperitoneal administration of nesfatin-1. The strongest evidence for vagal involvement in the anorexigenic effect of nesfatin-1 has been shown in a study with mice pretreated with capsaicin [46]. Capsaicin treatment caused the loss of capsaicin sensitive neurons [27], which have been shown to be activated by nesfatin-1 *in vitro [19]*. The capsaicin treatment prevented the reduction in food intake in response to nesfatin-1 administration [46]. This suggests that at least part of the anorexigenic effects of nesfatin is vagally mediated. However, whilst capsaicin treatment was originally believed to be an afferent lesioning tool there is now evidence to suggest efferent nerves are also affected [7]. Therefore the lack of effect of nesfatin-1 on food intake after capsaicin application could be due to altered sensory function or as a consequence of disrupted efferent function. Given the crude nature of this approach it remains to be conclusively determined whether the vagal afferents constitute an important route for nesfatin-1 to reduce food intake. Nonetheless, given the relative abundance of nesfatin-1 in the stomach (about 10 fold higher than the brain [48]) it would be logical for the vagus to be involved in the actions of nesfatin-1, similar to the action of other gastric peptides such as leptin [36] and ghrelin [12].

Our finding that nesfatin-1 inhibits tension receptor mechanosensitivity in HFD-induced obese mice suggests that in an obese state nesfatin-1 may increase food intake through the delaying of gastric satiety signals. However, this is doubtful as nesfatin-1 has been shown to reduce food intake in both leptin receptor deficient obese mice and HFD mice [45]. Additionally, intracerebroventricular administration of nesfatin-1 reduces food intake in both lean and obese rats [39]. Furthermore, nesfatin-1 reduced food intake through a reduction in meal size in lean rats whereas it increased inter-meal duration and reduced meal frequency in obese rats [39]. This suggests that diet induced obesity is associated with discrete changes in the anorexigenic effect of nesfatin-1. Tension receptors are involved in regulating acute food intake through the restriction of meal size. Thus, it is possible that whilst nesfatin-1 has a net anorexigenic effect in obese animals it may still influence meal size by inhibiting tension receptor mechanosensitivity. This is just speculation and the physiological significance of the nesfatin-vagal pathway needs to be addressed through further experiments. It is important to note that gastrointestinal vagal afferents are implicated in the regulation of numerous other functions. Therefore it remains a distinct possibility that the altered gastric vagal afferent response to nesfatin-1 may have no importance in the regulation of food intake, but instead be involved in the regulation of other vagally mediated functions within the gastrointestinal tract. For example, gastrointestinal peptides, via vagally mediated mechanisms, have been demonstrated to regulate gastric motility as well as gastrointestinal and pancreatic secretions [20, 29, 43]. In addition, alterations in vagal tone can impact on inflammation, respiration and heart rate [5, 38, 40] in response to a meal.

Gastric vagal afferent mechanosensitivity exhibits a circadian rhythmicity that is completely abolished in HFD induced obesity [22, 26]. The plasma levels of NUCB2/nesfatin-1 also appear to vary throughout the day [31]. In the current study, experiments were only performed within a short time range (0600-0900hr) to eliminate variation in mechanosensitivity due to this observed circadian rhythmicity. However, it has been demonstrated that the appetite hormone

leptin potentiates gastric mucosal receptor mechanosensitivity in a time of day dependent manner [26]. Therefore, it is possible that the effect of nesfatin-1 on gastric vagal afferent mechanosensitivity also varies throughout the day. This requires further investigation.

5. Conclusion

In conclusion, we show that the ability for nesfatin-1 to modulate gastric vagal afferent mechanosensitivity is sensitive to both over nutrition and deprivation. It still remains to be determined what the functional importance of this is in terms of regulation of food intake.

Conflict of Interest

The authors report no conflicts of interest.

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Figure legends

Figure 1: High fat diet feeding increases mouse weight and adiposity. (A) Proportional body weight gain over the 12 week diet period for mice that had either been fed a standard diet (SLD, ○) or a high fat diet (HFD, □). (B) Final body weight, (C) gonadal fat mass and (D) plasma nesfatin-1 levels in mice that were fed either SLD or HFD for 12 weeks or fasted overnight. (A) *P<0.05, ***P<0.001 vs. SLD, two-way ANOVA, Sidak post-hoc test. (B) and (C) ** P<0.01 vs. SLD, unpaired t-test (D) one-way ANOVA, P>0.05.

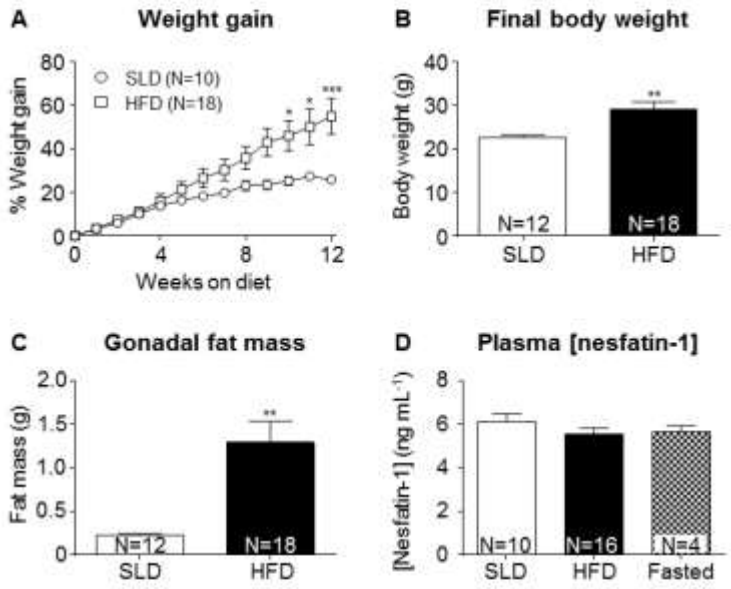

Figure 2: Nucleobindin 2 expression in the gastric mucosa and fat. The relative abundance of nucleobindin 2 mRNA present in the (A) gastric mucosa and (B) gonadal white adipose tissue in mice that were fed either the SLD (open bars), HFD (closed bars) or fasted overnight (checked bars).

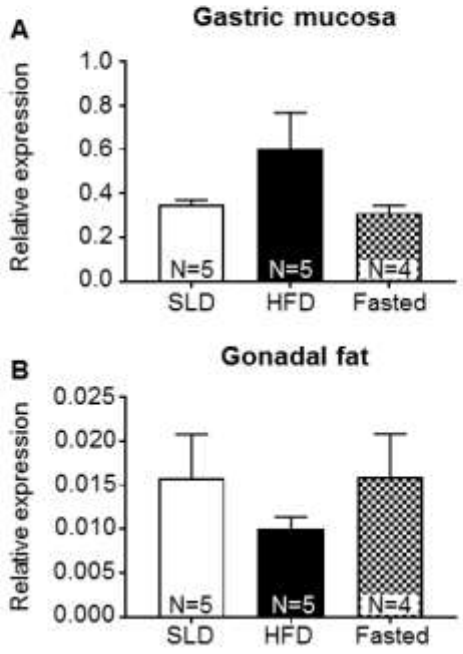

Figure 3: Nesfatin-1 potentiated gastric mucosal receptor mechanosensitivity in standard laboratory diet (SLD) fed and fasted mice. The responses of gastric mucosal receptors to mucosal stroking with calibrated von Frey hairs (10–1000mg) in the absence (○) and presence of nesfatin-1 30pM (□), 100pM (Δ) and 300pM (◊) from mice (A) fed a SLD, (B) fasted overnight or (C) fed a high fat diet (HFD). *P< 0.05, **P<0.01, ***P<0.001 vs. ○, two-way ANOVA, nesfatin-1 effect. (D) Diet significantly affected the potentiating action of nesfatin-1 on the response of mucosal receptors to mucosal stroking with a 200mg von Frey hair (**P<0.01, vs. HFD, two-way ANOVA, nutritional state effect).

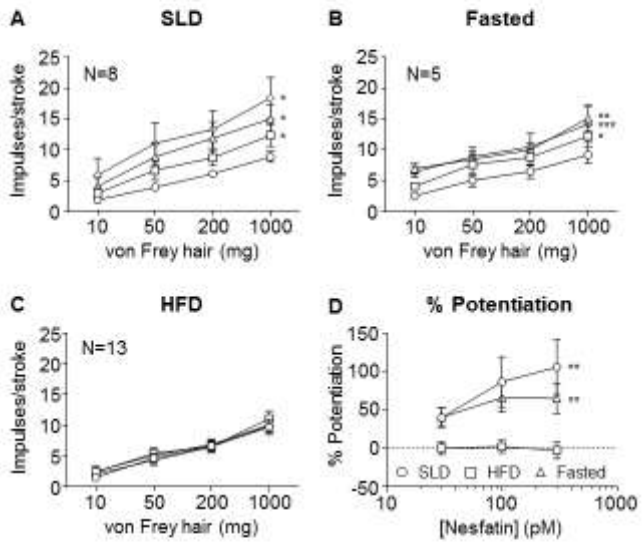

Figure 4: Nesfatin-1 gains an inhibitory effect on tension receptor mechanosensitivity in high fat diet (HFD) fed mice. The responses of gastric tension receptors to circular stretch with calibrated weights (1–5g) in the absence (○) and presence of nesfatin-1 30pM (□), 100pM (Δ) and 300pM (◊) from mice (A) fed a standard laboratory diet (SLD), (B) fasted overnight or (C) fed a HFD. *P< 0.05, vs. ○, two-way ANOVA, nesfatin-1 effect. (D) Diet significantly affected the inhibitory action of nesfatin-1 to the response to 3g stretch (D; *P<0.05, vs. SLD, two-way ANOVA, nutritional state effect).

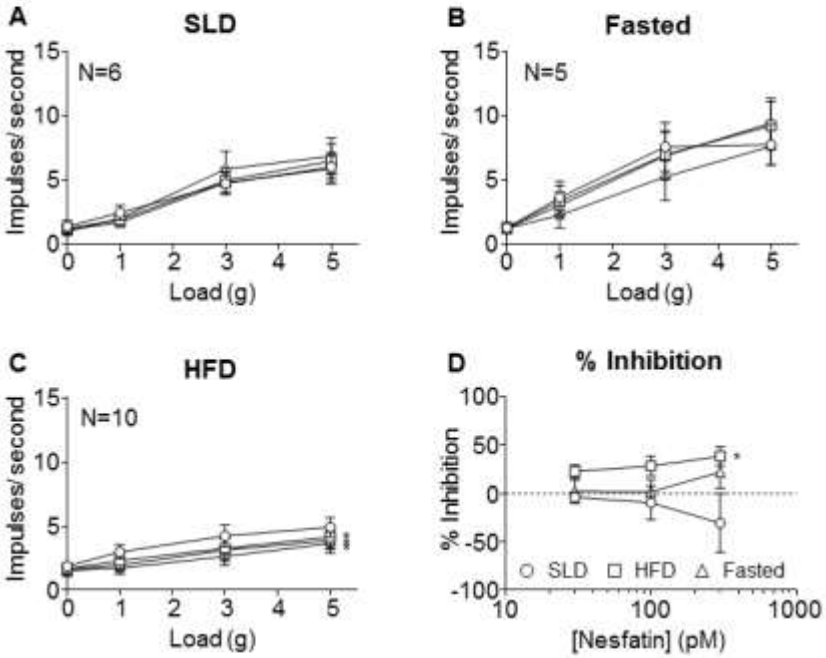

Table Legend

Table 1: Expression stability values of the candidate housekeeper genes β-actin (Actb), hypoxanthine guanine phosphoribosyl transferase (Hprt), β-2 microglobulin (B2m), 18S ribosomal (Rn18s) and peptidylprolyl isomerase A (Ppia) calculated using the Normfinder algorithm.*The lower the value the more stable the housekeeper gene.

	Rank	Gene	NormFinder stability value*
Stomach scrapings	1	Hprt	0.012
	2	Ppia	0.032
	3	B2m	0.042
	4	Actb	0.057
	5	Rn18s	0.131
White adipose tissue	1	Ppia	0.017
	2	Actb	0.039
	3	B2m	0.077
	4	Hprt	0.088
	5	Rn18s	0.097